

Endothelin Receptor Overexpression Alters Diastolic Function in Cultured Rat Ventricular Myocytes

Misuk Kang^{1,2}, Jeffery W. Walker^{1,3} and Ka Young Chung^{1,4,*}

¹Molecular and Cellular Pharmacology, University of Wisconsin, Madison, WI 53076, USA,

²Green Cross Corp., Yongin 446-770, Republic of Korea,

³Department of Physiology, University of Wisconsin, Madison, WI 53076, USA,

⁴School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea

Abstract

The endothelin (ET) signaling pathway controls many physiological processes in myocardium and often becomes upregulated in heart diseases. The aim of the present study was to investigate the effects of ET receptor upregulation on the contractile function of adult ventricular myocytes. Primary cultured adult rat ventricular myocytes were used as a model system of ET receptor overexpression in the heart. Endothelin receptor type A (ET_A) or type B (ET_B) was overexpressed by Adenoviral infection, and the twitch responses of infected ventricular myocytes were measured after ET-1 stimulation. Overexpression of ET_A exaggerated positive inotropic effect (PIE) and diastolic shortening of ET-1, and induced a new twitch response including twitch broadening. On the contrary, overexpression of ET_B increased PIE of ET-1, but did not affect other two twitch responses. Control myocytes expressing endogenous receptors showed a parallel increase in twitch amplitude and systolic Ca²⁺ in response to ET-1. However, intracellular Ca²⁺ did not change in proportion to the changes in contractility in myocytes overexpressing ET_A. Overexpression of ET_A enhanced both systolic and diastolic contractility without parallel changes in Ca²⁺. Differential regulation of this nature indicates that upregulation of ET_A may contribute to diastolic myocardial dysfunction by selectively targeting myofilament proteins that regulate resting cell length, twitch duration and responsiveness to prevailing Ca²⁺.

Key Words: Cardiac myocytes, Twitch kinetics, Diastolic dysfunction, Endothelin receptor, ET-1

INTRODUCTION

Endothelin-1 (ET-1) is known to exert physiological actions on many tissues including ventricular myocardium through two G-protein coupled receptor subtypes, endothelin receptor type A (ET_A) and type B (ET_B) (Kedzierski and Yanagisawa, 2001). In normal myocardium, 60-85% of ET-1 receptors are ET_A (Kobayashi *et al.*, 1999; Modesti *et al.*, 1999), and many physiological actions of ET-1 including its positive inotropic effect (PIE) are thought to be mediated mainly by ET_A (Endoh *et al.*, 1998; Goldberg *et al.*, 2000; Kelso *et al.*, 2000). ET_B activation may regulate contractile function under some circumstances (Saetrum Opgaard *et al.*, 2000; Piuholo *et al.*, 2003), but its precise role in myocardium remains uncertain.

ET-1 and ET receptors have been implicated in the pathophysiology of various cardiovascular disease states such as myocardial infarction, cardiac hypertrophy, and congestive heart failure (Yamamoto *et al.*, 2000a; Rich and McLaughlin, 2003). Previous studies have demonstrated increased levels

of ET-1, ET_A and in some cases ET_B in patients and in animal models of heart disease (Hasegawa *et al.*, 1996; Pönicke *et al.*, 1998; Pieske *et al.*, 1999; Zolk *et al.*, 1999; Asano *et al.*, 2002). Furthermore, a correlation between elevated ET-1 and diastolic dysfunction has been reported in humans (Napoli *et al.*, 1996) and in rats (Yamamoto *et al.*, 2000b). However, the precise mechanism of ET-1 related diastolic dysfunction is not fully understood yet.

Evaluating the biological actions of ET-1 *in vivo* represents a major challenge because it targets many tissues and therefore can result in complex systemic effects (Kobayashi *et al.*, 1999). Even within the heart, ET-1 targets vascular myocytes, endothelial cells and cardiac myocytes with complex and possibly regionally distinct outcomes. In diseased tissue, ET receptors can also change levels and patterns of expression (Kobayashi *et al.*, 1999; Yamamoto *et al.*, 2000a; Asano *et al.*, 2002). In recognition of this complexity, the present study was designed to evaluate the direct acute effects of ET-1 on contractile function of ventricular in which ET_A or ET_B have been

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*Corresponding Author

E-mail: kychung2@skku.edu

Tel: +82-31-290-7787, Fax: +82-31-292-8800

overexpressed. The data provide important new insights into the role of the ET signaling system in regulation of specific aspects of cardiac contractility including active relaxation and passive stiffness of ventricular myocardium. Importantly, delayed relaxation and increased passive stiffness are hallmarks of diastolic heart failure (Zile *et al.*, 2004) and cardiac dysfunction in sepsis (Chopra and Sharma, 2007). A better understanding of the involvement of ET signaling in diastolic heart failure and related conditions may lead to novel therapeutic strategies for this emerging class of heart disease.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless noted otherwise. Collagenase was from Worthington, Inc (Lakewood, NJ, USA). Complete protease inhibitor cocktail was from Roche (Mannheim, Germany). ET_A monoclonal antibody was from BD Transduction Laboratories (San Diego, CA, USA), X-rhod-1-AM ester was from Molecular Probes (Eugene, OR, USA), and other primary and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Isolation of adult rat ventricular myocytes

Animal handling practices have been approved by the Animal Care Committee of the University of Wisconsin. Ventricular cardiac myocytes were isolated from 3 month old male Sprague-Dawley rats by enzymatic digestion with collagenase as previously described (Pi *et al.*, 1997).

Primary culture and adenoviral infection of adult myocytes

Isolated myocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 1 mM CaCl₂, 5 mM taurine, 5 mM carnitine, and 5 mM creatine, plated on laminine-coated cover-slip, and infected with adenoviruses as previously described (Chung *et al.*, 2008).

Adenovirus constructs

Human ET_A or ET_B was fused to the N-terminus of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) in a pShuttle vector driven by the cytomegalovirus promoter (Stratagene, Garden Grove, CA, USA). Adenoviruses carrying protein constructs were generated using AdEasy adenoviral vector system (Stratagene, Garden Grove, CA, USA) according to the manufacturer's instructions. Viruses were amplified and purified by use of ViraKit AdenoMini-4 purification kit (Viraapur, LLC, San Diego, CA, USA).

Measurement of ET_A or ET_B distribution and overexpression level

Images of ET_A or ET_B overexpressed myocytes were obtained using on a Bio-Rad Radiance 2100 laser scanning confocal microscope with data acquisition controlled by Lasersharp 2000 software. Quantitative analyses of Perinuclear regions, sarcolemma, and T tubules were performed with National Institutes of Health ImageJ software. The overexpression levels of ET_A and ET_B were analyzed as described previously (Chung *et al.*, 2008). Briefly, the radiance confocal

microscope was calibrated by the use of a purified bacterial expressed CFP or YFP construct provided by Dr. Dapankar Battacharaya. The concentration of the standard CFP or YFP protein was estimated by a bicinchoninic acid protein assay and by Coomassie blue binding on a SDS-PAGE gel using bovine serum albumin as a protein standard. With the use of the same microscope settings for the acquisition of cell images (laser power, pin hole dimensions, black levels, objective, etc.), the mean fluorescence was measured as a function of the concentration CFP or YFP standard in the solution. The mean fluorescence expressed within ventricular myocytes was then used to estimate the effective fluorescence concentration by comparison with the CFP or YFP standard curve. The endogenous ET_A concentration was estimated at 106 receptor sites/30 pl per myocyte (37, 40; unpublished data) or 55 nM, such that the levels of overexpression were 12- and 10-fold in ET_A-CFP and ET_B-YFP overexpressed myocytes, respectively.

Twitch measurements

Myocytes were analyzed in 1 mM Ca²⁺ Ringer's solution (125 mM NaCl, 5 mM KCl, 2 mM NaH₂PO₄, 5 mM sodium pyruvate, 1.2 mM MgSO₄, 11 mM glucose, 0.5 mM CaCl₂, 25 mM HEPES, pH 7.4) unless otherwise noted. Cell twitches were initiated by electric field stimulation with a SD9 stimulator (Grass Technologies, West Warwick, RI, USA) in a modified PH1 chamber (Warner Instruments, Hamden, CT, USA) mounted on a Nikon Diaphot inverted microscope. The stimulation protocol was 0.5 Hz, 10 ms duration, and 50 V at 21°C. Individual myocytes were monitored with a model VED 104 video edge detector (Crescent Electronics, Sandy, UT, USA) and cell shortening was recorded using Felix software (Photon Technology International, Birmingham, NJ, USA).

Confocal imaging and Ca²⁺ measurements

Confocal microscopy was performed on a Bio-Rad Radiance 2100 laser scanning confocal microscope with data acquisition controlled by Lasersharp 2000 software. Intracellular Ca²⁺ was monitored with the Ca²⁺ dye X-rhod-1 excited at 543 nm with a Green Helium Neon laser and emitted fluorescence collected at 600 nm recorded in line scan mode at 2 msec/line. The data acquisition line was oriented along the long axis of the cell. Myocyte shortening was monitored using the raw line scan data and analyzed with Image J software. Line scan images were analyzed by IDL software to obtain fluorescence changes associated with relative changes in Ca²⁺. Culture media was replaced with 1 mM Ca²⁺ Ringer's solution, and 2 µM X-rhod-1-AM was loaded for 15 min at room temperature. After washing out X-rhod-1, myocytes were stimulated electrically as described above. Potential damage from laser excitation of X-rhod-1 did not adversely affect contractility as responses were indistinguishable with and without X-rhod-1 or with laser illumination versus video edge detection.

Statistics

Data are expressed as mean ± sem and analyzed using an unpaired student's *t*-test, and a one-way ANOVA (where appropriate). Values of *p* < 0.05 were considered to be significant for all statistical tests. Correlations between any two parameters were characterized initially by the slope of a regression line through the raw data. The existence of significant correlations was then evaluated by Pearson correlation coefficient (1.0 to -1.0) and *p* value by use of the commercial software

Minitab 14 (State college, PA, USA). A value of $p < 0.005$ was taken to be a significant correlation, and Pearson correlation values between 0.4 to -0.4 were taken to be only weak correlations despite in some cases being statistically significant.

RESULTS

Overexpression of ET_A or ET_B in adult rat ventricular myocytes

ET_A or ET_B were C-terminally tagged with CFP or YFP to monitor their expression in myocytes, which was typically ro-

bust after 40-48 hrs of adenoviral infection (Fig. 1). After 48 hours of adenoviral infection, ET_A-CFP was expressed to a level of 12-fold over endogenous ET_A, and ET_B-YFP was expressed to a level of 10-fold. Physiological responses of myocytes were evaluated within this 40-48 hour window of infection to minimize the impact of structural remodeling that can occur in cultured adult rat myocytes (Chung *et al.*, 2008).

ET_A-CFP was overexpressed ET_A and localized at the perinuclear region (18±3% of CFP fluorescence), sarcolemma (31±5%), and transverse-tubules (T-tubules; 52±6%) (Fig. 1A). ET_B-YFP was overexpressed and localized more at the perinuclear region (44±9% of YFP fluorescence), less in the sarcolemma (8±5%) and about the same in T-tubules (48±8%) (Fig. 1B). In earlier studies, we presented evidence that T-tubule localized ET_A was primarily responsible for regulation of myocyte contractility by ET-1 (Chung *et al.*, 2008). Here we examined the functional consequences of ET-1 stimulation in myocytes overexpressing ET receptors by measuring additional twitch and Ca²⁺ handling parameters as described below.

Evaluation of contractile responses to ET-1

Fig. 2A illustrates the three twitch parameters of adult rat ventricular myocyte in response to ET-1. PIE was determined as % increase in twitch amplitude, diastolic shortening was determined as % change in cell length in diastole, and twitch duration was evaluated by measuring changes in the twitch width at 50% of maximal twitch amplitude (Fig. 2A).

In freshly isolated myocyte, ET-1 induced PIE and slight increase in diastolic shortening, but no changes in twitch duration. The increased PIE and diastolic shortening was abolished by ET_A specific antagonist, BQ123 (data not shown).

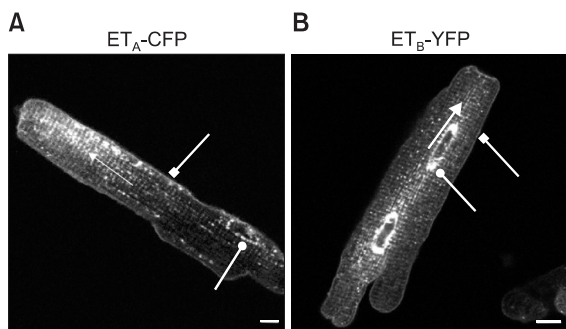


Fig. 1. Confocal fluorescence images of myocytes overexpressing ET_A or ET_B receptors. Cultured adult rat myocytes were infected with adenovirus vectors expressing receptors ET_A-CFP (A) or ET_B-YFP (B) and then imaged after 40 hours in culture. Perinuclear regions are indicated with round arrow head, sarcolemma with square arrow head, and T-tubules with arrows. Scale bars = 10 μm.

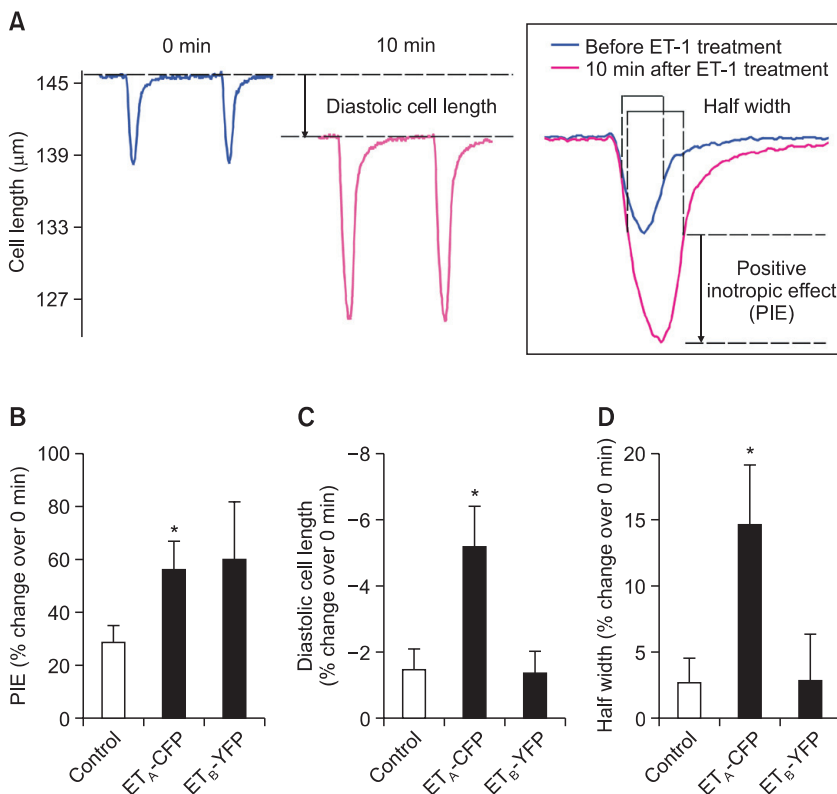


Fig. 2. ET-1-induced twitch responses of myocytes overexpressing ET_A or ET_B receptors. (A) Representative twitch trace after ET-1 stimulation. Electrically paced twitches were measured before (blue traces) and 10 min after (pink traces) treatment with 10 nM ET-1. (B-D) ET-1-induced twitch responses in control, ET_A-CFP, and ET_B-YFP overexpressed myocytes are compared for PIE (B), diastolic shortening (C), and twitch duration (D). *Indicates statistically significant differences compared to control with $p < 0.05$, $n \geq 4$.

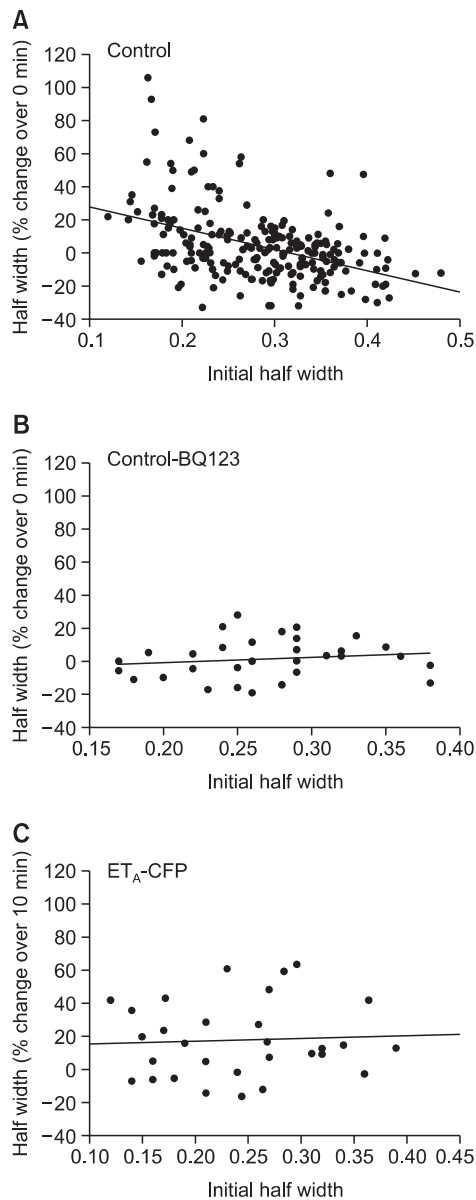


Fig. 3. Dependence of ET-1-induced twitch prolongation on initial twitch duration. ET-1-induced twitch prolongation was plotted against initial twitch duration for each cell, and the data were analyzed by linear regression. (A) Twitch prolongation versus initial twitch half-width in control cells. (B) Twitch prolongation versus basal twitch half-width in the presence of 1 μ M BQ-123 in control cells. (C) Twitch prolongation versus basal twitch half-width in ET_A-CFP overexpressed cells.

ET_B specific agonist s6c (sarafotoxin) did not induce any of the three twitch responses (data not shown). These data suggest that at the normal expression level of ET_A and ET_B ET-1 increases PIE and diastolic shortening via ET_A.

In control myocytes expressing green fluorescent protein (GFP) alone or myocytes that were non-virus infected, 10 nM ET-1 induced a significant increase in PIE and a slight increase in diastolic shortening (Fig. 2B-D). Interestingly, in this data set, control myocytes exhibited a $2.4 \pm 2.2\%$ increase in twitch duration following ET-1 stimulation (Fig. 2D), but this

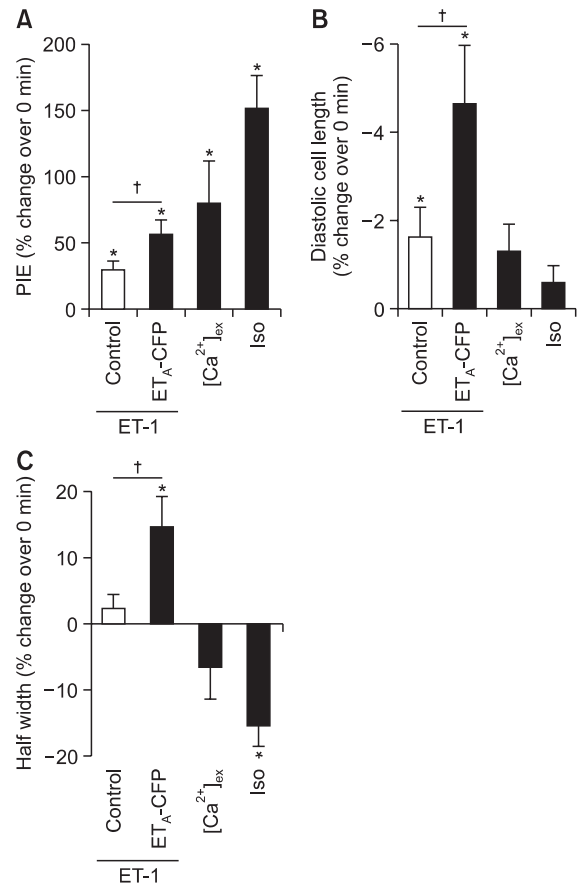


Fig. 4. Myocyte twitch responses to different inotropic stimuli. Responses to 10 nM ET-1 in control and ET_A-CFP overexpressing myocytes are compared to responses in control myocytes to a change in extracellular Ca²⁺ ([Ca²⁺]_{ex}) from 1 mM to 2 mM Ca²⁺, or to 10 nM Isoproterenol (Iso). PIE (A). Diastolic cell shortening (B). Twitch duration (C). *Indicates significantly different compared to before treatment with $p < 0.05$. †Indicates statistical significance with $p < 0.05$, $n \geq 8$.

was not statistically significant across the entire myocyte population due to the cell to cell variability (discussed below).

Upregulation of ET_A-CFP significantly increased the PIE and dramatically exaggerated diastolic cell shortening (Fig. 2B-D). These myocytes also showed an increase in twitch duration in response to 10 nM ET-1 (Fig. 2D). For comparison, ET_B-YFP was expressed in myocytes under similar conditions. Stimulation with 10 nM ET-1 induced an increase in twitch amplitude (PIE), but this response was not accompanied by changes in diastolic shortening or twitch duration (Fig. 2B-D). Thus contractile parameters subject to be controlled by the ET system depended upon the receptor subtype expressed. Hereafter, we focused on the effect of ET_A-CFP overexpression in ventricular myocytes.

Cell to cell variability and correlations among twitch parameters

In an attempt to better understand the variability in twitch duration properties, we searched for correlations among twitch parameters on a cell by cell basis.

There was a negative correlation between the twitch dura-

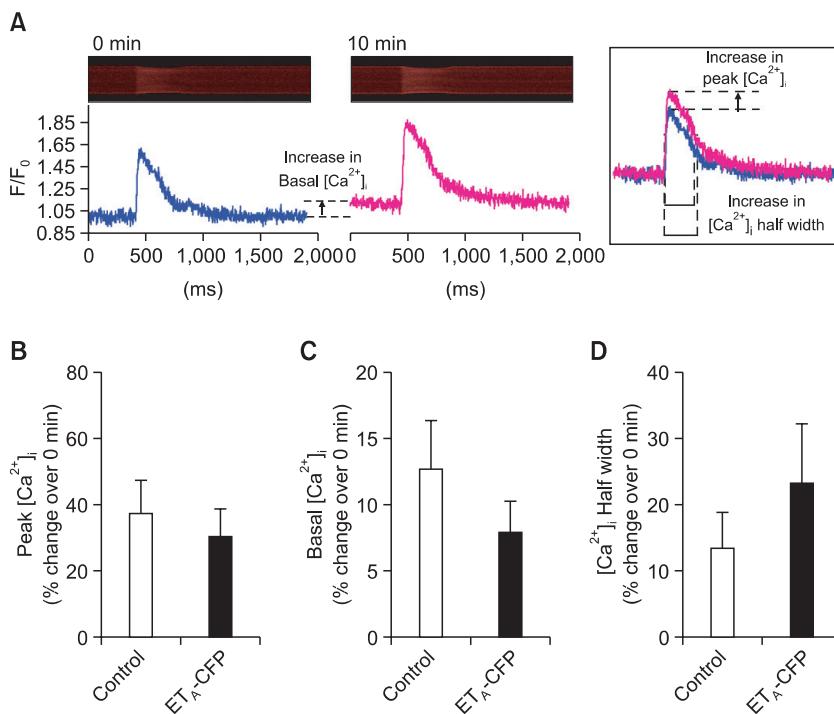


Fig. 5. ET-1-induced Ca²⁺ transient in ET_A overexpressing cardiac myocytes. (A) Representative Ca²⁺ transient after ET-1 stimulation. Confocal line scans (top panels) from which Ca²⁺ transients (bottom panels) were derived for a myocyte before (blue traces) and 10 min after (pink traces) 10 nM ET-1. (B-D) Ca²⁺ responses in control and ET_A-CFP overexpressed myocytes are compared for peak systolic Ca²⁺ (B), diastolic Ca²⁺ (C), and Ca²⁺ duration (width at half peak amplitude) (D), n≥13.

tion before ET-1 treatment and the % change in twitch duration after ET-1 treatment ($P_{\text{corr}} = -0.468$, $p < 0.001$; Fig. 3A). This observation indicated that in control myocytes, 10 nM ET-1 produced twitch broadening when the initial twitch duration was relatively short. In contrast, such a dependence on the basal twitch duration was not observed for the PIE ($P_{\text{corr}} = -0.065$, $p = 0.393$) or the diastolic shortening response ($P_{\text{corr}} = -0.181$, $p = 0.08$) (Data not shown).

The observed negative correlation between basal twitch duration and change in twitch duration was abolished by ET_A antagonist treatment (BQ123) (Fig. 3B) indicating that ET-1-induced twitch prolongation in sub-population of cells was through ET_A. The negative correlation was also abolished in cells overexpressing ET_A-CFP (Fig. 3C) inducing entire population of cells to show prolonged twitch duration upon ET-1 treatment.

Twitch responses to other stimuli

To determine whether these changes in twitch behavior were unique to ET-1, twitch responses to two common inotropic interventions were determined: i) elevated extracellular Ca²⁺ concentration ([Ca²⁺]_o), and ii) 10 nM isoproterenol (Iso). Increasing [Ca²⁺]_o from 1 mM to 2 mM resulted in an increase in twitch amplitude (Fig. 4A), whereas the diastolic cell length and twitch duration were not significantly altered (Fig. 4B and 4C). Iso increased twitch amplitude and promoted a decrease in twitch duration, but did not significantly alter diastolic cell length (Fig. 4). These results indicated that diastolic cell shortening and prolonged twitch duration were unique responses of rat myocytes to ET-1, and not shared with Iso or elevated [Ca²⁺]_o.

Evaluation of Ca responses to ET-1

To study ET-1 actions on Ca²⁺ amplitudes and kinetics, three main parameters of Ca²⁺ transients were analyzed: sys-

tolic Ca²⁺, basal (diastolic) Ca²⁺, and duration of the Ca²⁺ transient (Fig. 5A). Changes in intracellular systolic and diastolic Ca²⁺ generally changed in parallel with contractility in control myocytes (compare Fig. 5A with Fig. 2A). However, ET_A-CFP overexpression did not alter the intracellular Ca²⁺ changes compared to control myocytes (Fig. 5B-D). In short, ET_A-CFP overexpression had pronounced effects to exaggerate contractile responses and had virtually no effect to enhance the corresponding Ca²⁺ responses.

DISCUSSION

To our knowledge, this is the first study to examine the effects of ET_A or ET_B overexpression on twitch behavior and Ca²⁺ handling in isolated adult ventricular myocytes. The major findings of the present study can be summarized as follows: 1) contractile responses to ET-1 including PIE, diastolic cell shortening and prolongation of twitch duration were exaggerated by ET_A overexpression, but underlying Ca²⁺ responses were not, and 2) myocytes overexpressing ET_B responded to ET-1 with an increase in PIE only. Overall, the data provide support for the view that under some conditions the myocardial ET system can preferentially regulate contractility in diastole versus systole. It is also apparent that ET signaling can regulate contractility and [Ca²⁺]_i somewhat independently, possibly by regulating the coupling between contraction and [Ca²⁺]_i via direct actions on the myofilaments. These effects on myocardial contractility were also shown to be unique to ET-1 as they were not observed for the β-adrenergic agonist isoproterenol or in response to elevated extracellular Ca²⁺.

Two contractile parameters, PIE and prolongation of twitch duration in response to ET-1 are already reported in previous studies with species variation. ET-1-induced PIE is widely observed in many species including rabbit (Yang *et al.*, 1999),

human (Saetrum Opgaard *et al.*, 2000), rat (Kelly *et al.*, 1990; Pi *et al.*, 1997) and ferret (Wang *et al.*, 1991). ET-1-induced prolongation of twitch duration is also reported in human (Goldberg *et al.*, 2000), rabbit (Li *et al.*, 1991; Yang *et al.*, 1999) guinea pig (Prendergast *et al.*, 1997), ferret (Evans *et al.*, 1994), mouse ventricular myocytes (Pi *et al.*, 2002), and in failing dog heart (Heineke and Molkentin, 2006), but *not in rat myocytes* (Westfall *et al.*, 2005).

Consistent with previous studies in rat myocardium, ET-1 induced significant increase in PIE, and we did not detect statistically significant prolongation of the twitch duration in all data sets *averaged over the entire population of control adult rat ventricular myocytes*. However, portion of cells displayed clear ET-1 dependent changes in twitch duration. By searching for correlations among twitch parameters on a cell by cell basis, we detected an intriguing and previously unrecognized relationship between basal twitch duration and % change in duration after ET-1. One potential explanation for such a correlation is the existence of subpopulations of cells characterized by different initial twitch duration and distinct responsiveness to ET-1. Subpopulations of myocytes might be explained by differences of electrical and mechanical properties of ventricular myocytes derived from different regions of the heart (Stones *et al.*, 2008). The potential for regional differences in myocardial responses to ET-1 as suggested by the present data remains speculative however, and needs to be explored further. ET_A overexpression shifted all myocytes toward a prolonged twitch duration following agonist treatment resulting in a significant increase in mean twitch duration and a loss of correlation between initial twitch duration and twitch prolongation.

A relatively underexplored physiological parameter evaluated here was diastolic shortening. How G-protein coupled receptor stimulation affects diastolic cell length has not been widely discussed in the literature. In many reports, representative twitch traces showed clear diastolic cell shortening (Kohmoto *et al.*, 1993; Yang *et al.*, 1999) or increases in resting tension (Volkman *et al.*, 1990) but this phenomenon is typically not quantified or pursued further. The present study reports for the first time that ET-1 induces diastolic cell shortening which is exaggerated upon ET_A up-regulation.

A heart in failure is unable to deliver an adequate cardiac output either due to ventricular systolic dysfunction, ventricular diastolic dysfunction, or both. Systolic dysfunction is associated with reduced contractility, whereas diastolic dysfunction arises from abnormalities in diastolic distensibility, filling or relaxation of the ventricles (Aurigemma *et al.*, 2006). Elevated ET-1 correlates with diastolic dysfunction in humans (Napoli *et al.*, 1996) and rats (Yamamoto *et al.*, 2000b). Previous studies with animal models revealed that treatment with ET receptor antagonists attenuated left ventricular end diastolic pressure and prevented or delayed the onset of heart failure (Mulder *et al.*, 1997; Moe *et al.*, 1998; Onishi *et al.*, 1999). Diastolic dysfunction associated with sepsis (Chopra and Sharma, 2007) and with exercise intolerance in heart failure patients (Cheng *et al.*, 2001) may also be related to upregulation of ET-1 signaling. However, the precise mechanism of ET-1-related diastolic dysfunction is not studied yet.

The present results are consistent with the possibility that up-regulation of ET_A expression may have adverse effects on the heart by contributing disproportionately to diastolic dysfunction. Increased diastolic cell shortening would ultimately

be detrimental by increasing left ventricular end diastolic pressure. The observation of this study that ET-1 induced the diastolic shortening might explain the report that ET-1 treatment increased left ventricular end diastolic pressure in isolated rat hearts (Ebihara *et al.*, 1996). Furthermore, excessive twitch prolongation could also be detrimental by significantly compromising filling time. We suggest that combination of increase in these two contractile parameters upon up-regulation of ET_A would have detrimental effects in diastolic dysfunction. An important next step will be to examine the functional impact of altered expression of ET_A and ET_B in cardiac cells from diseased hearts, with the added challenge of defining disease stage and etiology as unambiguously as possible.

In conclusion, our findings reveal that ET_A overexpression exaggerated three contractile properties in rat ventricular myocytes: twitch amplitude, diastolic cell shortening and twitch prolongation. In contrast, ET_A overexpression did not exaggerate systolic Ca²⁺ amplitude, time course or diastolic Ca²⁺ levels. This difference suggests the intriguing possibility that increased levels of ET_A expression in heart failure may confer benefit to the heart by increasing contractile performance without increasing Ca²⁺ cycling. Conversely, ET-1 signaling through ET_A may also contribute to detrimental changes in cardiac function by: i) promoting diastolic cell shortening (thereby increasing myocardial stiffness/decreasing distensibility) and ii) delaying relaxation (thereby compromising filling). Intriguingly, these ET-1/ET receptor effects alter myocyte contractility in a manner that is reminiscent of myocardial performance in diseased hearts, specifically increased contractility (stiffness) in diastole and prolonged contraction time in systole. Viewing these results optimistically, it may be that diastolic parameters are regulated independently enough from systolic parameters by ET-1 signaling, that selective blockade of detrimental aspects of ET-1 overstimulation may be a therapeutically viable strategy against the emerging problem of diastolic heart failure.

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